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# Acute regulation of 5′-AMP-activated protein kinase by long-chain fatty acid, glucose and insulin in rat primary adipocytes

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## INTRODUCTION

This section provides the following. (i) Figures S1 and S2 that supplement the introduction of the paper by summarizing the major pathways of glucose and fatty acid metabolism in rat adipocytes (see these Figure legends for references [1–3]). (ii) A set of calculations of the rates of glucose-related metabolic processes in adipocytes were developed. (iii) Those calculations were applied to data from the 1972 paper of Saggerson [4] that showed the effects of a range of concentrations of palmitate and adrenaline on adipocyte glucose metabolism. This treatment allowed some further interpretations of the data of [4] that is relevant to the discussion section of the paper. It also provided the basis for comparisons of the effects of palmitate and adrenaline on adipocyte glucose metabolism.

## METABOLIC FLUX CALCULATIONS

### Definitions

FA, GG, L, P and TCA (trichloroacetic acid) indicate the number of $\mu$g atoms of glucose carbon converted to fatty acid, glyceride glycerol, lactate, pyruvate and TCA cycle $CO_2$ respectively.

### Method 1: an empirical calculation of the rate of glucose utilization

Studies by [4] reported rates of conversion of glucose into FA + GG + L + P by rat adipocytes without providing any measurements of $CO_2$ formation. Therefore an empirical method was devised that allowed $CO_2$ formation to be calculated from those data thereby allowing calculation of the overall rate of glucose utilization which (see Figure S1) approximates to:

$$\text{Glucose utilization} = \text{FA} + \text{GG} + \text{L} + \text{P} + \text{CO}_2 \qquad (1.1)$$

This is where:

$$\text{CO}_2 = \text{PPP CO}_2 + \text{PDH CO}_2 + \text{TCA cycle CO}_2 \qquad (1.2)$$

To develop this method, we used the raw experimental data from studies by Saggerson and Greenbaum [5,6], who incubated pieces of rat epididymal adipose tissue and measured PPP (pentose phosphate pathway) $CO_2$, PDH $CO_2$ and TCA cycle $CO_2$ as well as total glucose utilization and formation of FA, GG, L and P (all expressed relative to g of wet weight of tissue). Figure S3 shows that a plot of PPP $CO_2$ against FA is linear with a gradient of 0.26 and an intercept of 1.83 on the PPP $CO_2$ axis. Therefore PPP $CO_2$ formation was calculated as:

$$\text{PPP CO}_2 = (0.26\text{FA}) + 1.83 \qquad (1.3)$$

A similar linear relationship between PPP $CO_2$ and FA can be deduced for adipocytes from the data of [7]. Also, using data for adipocytes incubated with insulin and either 2.5 or 5 mM glucose (raw data from [8] and unpublished results) a linear plot similar to Figure S3 has a gradient of 0.265 ($n = 14$, $r = 0.992$). For epididymal fat pieces or adipocytes, the formation of TCA cycle $CO_2$ from glucose is low unless NEFA (non-esterified fatty acids) is added to the incubations or is generated *in situ* in response to a lipolytic agent. In those instances, esterification or re-esterification of NEFA leads to a large increase in GG formation from glucose together with a large increase in TCA cycle activity to fuel the provision of the ATP for NEFA esterification and re-esterification. When a spread of values is obtained in this way Figure S4 shows that a plot of TCA cycle $CO_2$ against GG is linear with a gradient of 0.61 and a positive intercept of 2.92 on the GG axis. Therefore TCA cycle $CO_2$ formation was calculated as:

$$\text{TCA CO}_2 = 0.61\text{GG} - 2.92 \qquad (1.4)$$

On the basis that acetyl-CoA derived from glucose by adipocytes is only used for the formation of FA and TCA cycle $CO_2$, the $\mu$mol of PDH $CO_2$ is 50% of the $\mu$g atoms of carbon converted into FA and TCA cycle $CO_2$ and therefore:

$$\text{PDH CO}_2 = 0.5 \times (\text{FA} + 0.61\text{GG} - 2.92) \qquad (1.5)$$

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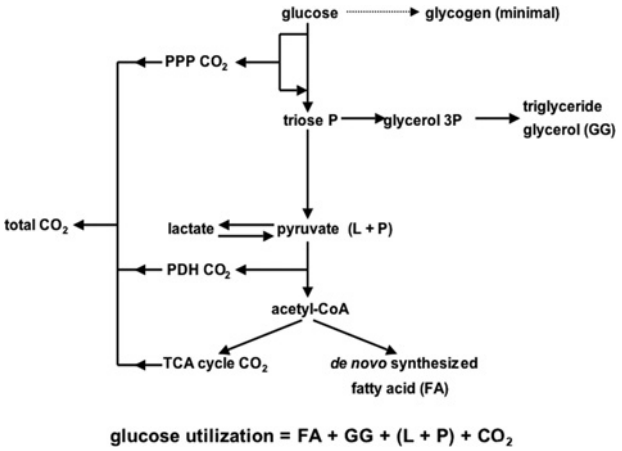


**Figure S1 The adipocyte glucose economy**
Based on the findings of [1] the products of glucose metabolism by rat white adipocytes can be considered to be almost entirely *de novo* synthesized FA, the glycerol moiety of TAG (GG), $CO_2$ (from the PPP, PDH and the TCA cycle) and accumulated lactate (L) and pyruvate (P). Synthesis of glycogen makes only a minor contribution to glucose utilization by adipocytes.

Substituting eqn (1.3), (1.4) and (1.5) into (1.2):

$$\text{Total } CO_2 = 0.76\text{FA} + 0.92\text{GG} + \text{L} + \text{P} - 2.6 \qquad (1.6)$$

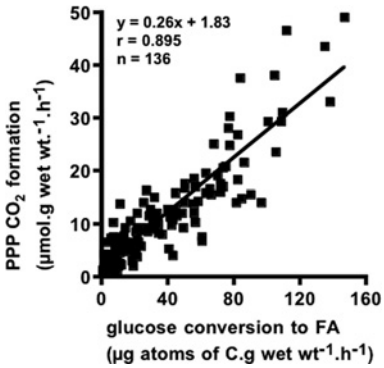


**Figure S3 The relationship between glucose conversion into pentose phosphate pathway $CO_2$ and fatty acid formation**
The data are for rat epididymal adipose tissue pieces and are taken from [5,6]. Each point is for a separate incubation.

Substituting eqn (1.6) into eqn (1.1):

$$\text{Glucose utilization} = 1.76\text{FA} + 1.92\text{GG} + \text{L} + \text{P} - 2.6 \qquad (1.7)$$

The value of $-2.6\ \mu$g atoms of C per g of wet weight is very small compared with the summation of the other values (see Figure S5) and therefore can be ignored. This reduces eqn (1.7) to eqn (1.8), which is not limited solely to use with data expressed per g of wet weight and can be used with data expressed in other

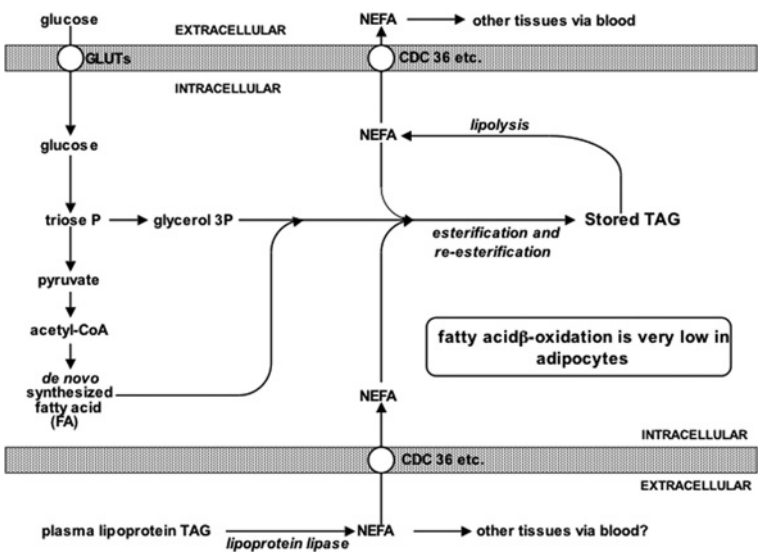


**Figure S2 The adipocyte fatty acid economy**
NEFAs from three sources are precursors of the rat white adipocyte's store of TAG. These NEFAs originate from: (i) the action of lipoprotein lipase in the adipose tissue capillary bed followed by transport of NEFA into the cell; (ii) intracellular *de novo* synthesis of FA; (iii) re-esterification of some of the NEFA produced in lipolysis with the remainder exiting the cell and being removed by blood flow. To provide glycerol 3-phosphate for NEFA esterification/re-esterification GLUT4 is the predominant transporter of glucose together with some contribution by GLUT1. Influx and efflux of NEFA are envisaged as predominantly being facilitated by CDC 36 and possibly by other NEFA transporters. The arrows indicate the directions of net NEFA fluxes. Under high insulin conditions (e.g. feeding) esterification and re-esterification predominate over lipolysis whereas under low insulin conditions (e.g. fasting and diabetes) lipolysis predominates. The rate of fatty acid $\beta$-oxidation makes only a very small contribution to overall NEFA metabolism in white adipocytes [2,3].

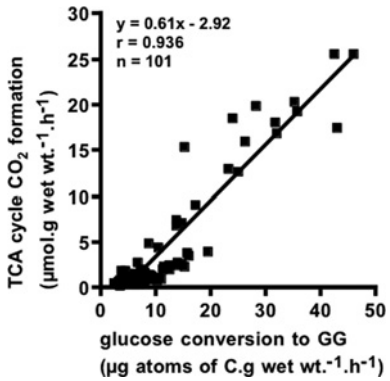


**Figure S4 The relationship between glucose conversion to TCA cycle $CO_2$ and glyceride glycerol formation**
The data are from the same experiments as in Figure S3. Each point is for a separate incubation.

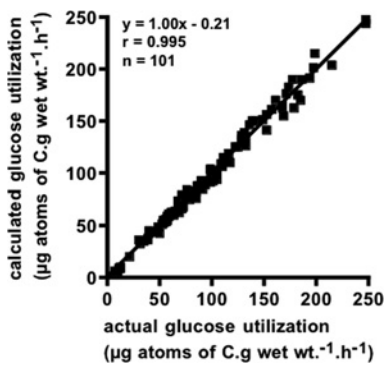


**Figure S5 The correlation between calculated rates of glucose utilization and directly measured rates**
The data are from the same experiments as in Figures S3 and S4. Each point is for a separate incubation. Direct measurement of glucose utilization was as for eqn (1.1). Eqn (1.8) was used to calculate glucose utilization without the use of experimental measurements of $CO_2$ formation.

ways (e.g. relative to DNA or protein):

$$\text{Glucose utilization} = 1.76\text{FA} + 1.92\text{GG} + \text{L} + \text{P} \qquad (1.8)$$

Equation (1.8) provides Method 1 for calculation of glucose utilization and is validated by Figure S5 that shows a linear plot of calculated glucose utilization against actual measured glucose utilization (total lipid + total $CO_2$ + L + P) with a gradient of 1.00 and passing very close to the graph origin. Other similar linear plots provided further validation of the method. These used data for epididymal fat pieces from [1,9] (gradient = 0.994, $n = 5$, $r = 0.989$) and from [10] (gradient = 0.999, $n = 4$, $r = 0.995$) as well as for isolated adipocytes from [7] (gradient = 0.983, $n = 5$, $r = 0.981$) and from [10] (gradient = 1.009, $n = 14$, $r = 0.995$).

## Method 2: calculation of the rate of glucose utilization

This provided an alternative method for calculation of glucose utilization from the FA + GG + L + P values found in [4]. It additionally provided the basis for estimations of the contributions of various metabolic processes as shown below. The following assumptions are made. (i) ATP utilization for the conversion of glucose carbon into lipid (FA + GG) equals glucose metabolism to provide this ATP by oxidative and substrate level phosphorylations. (ii) The average chain length of FA formed from glucose carbon is C-17 (approximately 50% C-16 and 50% C-18). (iii) All of the cytosolic acetyl-CoA used for FA synthesis is formed by ATP citrate lyase. (iv) 0.26 $\mu$mol of PPP $CO_2$ (equivalent to 0.52 $\mu$mol of PPP NADPH) are produced per $\mu$g atom of glucose carbon incorporated into FA. (v) NADPH for FA synthesis is provided solely by the PPP or from cytosolic NADH via the concerted actions of cytosolic NAD-malate dehydrogenase and NADP-malate dehydrogenase. (vi) The reoxidation of each $\mu$mol of NADH or $FADH_2$ by the mitochondrial respiratory chain coupled to oxidative phosphorylation gives rise to 2.5 or 1.5 $\mu$mol of ATP respectively. (vii) Mitochondrial/cytosolic shuttling of reducing equivalents only involves NAD-linked redox reactions, i.e., glycerol phosphate shuttle activity is insignificant.

Values are $\mu$mol of ATP, NADH or $FADH_2$ per $\mu$g of atom of glucose C in a product.

## ATP utilization for FA synthesis

(i) Hexokinase + phosphofructokinase = 0.5FA; (ii) ACC = 7.5FA/17 = 0.441FA; (iii) ATP citrate lyase = 8.5FA/17 = 0.5FA; (iv) pyruvate carboxylase = 6.16FA/17 = 0.362FA. Synthesis of 1 $\mu$mol of C-17 fatty acid requires 15 $\mu$mol of NADPH. 8.84 (0.52×17) $\mu$mol of this NADPH is provided by the PPP (see above) leaving 6.16 $\mu$mol to be provided by NADP-malate dehydrogenase. That reaction depletes oxaloacetate/malate necessitating an equivalent anaplerotic replenishment by the ATP-dependent pyruvate carboxylase reaction.

$$\text{TOTAL} = 1.803\text{FA} \qquad (2.1)$$

## ATP utilization for GG formation

(i) Hexokinase + phosphofructokinase = 0.333GG; (ii) Fatty acyl-CoA synthetase = 2GG:

$$\text{TOTAL} = 2.333\text{GG} \qquad (2.2)$$

Summing eqn (2.1) and eqn (2.2), ATP utilization for FA + GG formation is:

$$1.803\text{FA} + 2.333\text{GG} \qquad (2.3)$$

## ATP formation by substrate level phosphorylations

(i) Formation of accumulated lactate and pyruvate = 0.333P + 0.333L; (ii) Formation of pyruvate subsequently converted into acetyl-CoA = 0.5FA + 0.5TCA; (iii) TCA cycle

(1 GTP per 2 $CO_2$) = 0.5TCA:

$$\text{TOTAL} = 0.333\text{P} + 0.333\text{L} + 1.0\text{TCA} + 0.5\text{FA} \quad (2.4)$$

## ATP formation by oxidative phosphorylation following reoxidation of cytosolic reducing equivalents by the respiratory chain

The following processes result in formation (F) of cytosolic NADH. (i) Glucose → accumulated pyruvate = 0.333P; (ii) Glucose → pyruvate subsequently converted into acetyl-CoA = 0.5FA + 0.5TCA. The following processes result in utilization (U) of cytosolic NADH: (iii) reduction of dihydroxyacetone phosphate to glycerol 3-phosphate = 0.333GG; (iv) The concerted actions of NAD- and NADP-malate dehyrogenases to provide NADPH for FA synthesis = 0.36FA. Formation of lactate involves no net formation of NADH and is omitted from this calculation.

Net cytosolic NADH balance (F − U) is:

$$0.333\text{P} + 0.5\text{FA} + 0.5\text{TCA} - 0.333\text{GG} - 0.36\text{FA} \quad (2.5)$$

Reoxidation of this by the respiratory chain/oxidative phosphorylation yields ATP as:

$$0.833\text{P} + 0.35\text{FA} + 1.25\text{TCA} - 0.833\text{GG} \quad (2.6)$$

## ATP formation by oxidative phosphorylation following reoxidation of mitochondrial reducing equivalents by the respiratory chain

(i) The PDH NADH yield is 0.5FA + 0.5TCA which is equivalent to 1.25×(FA + TCA) ATP; (ii) the TCA cycle yields of NADH and $FADH_2$ are 1.5TCA and 0.5TCA, respectively yielding (3.75 + 0.75)TCA ATP. Therefore formation of ATP by mitochondrial processes is:

$$1.25\text{FA} + 5.75\text{TCA} \quad (2.7)$$

If ATP utilization is matched by ATP formation then eqn (2.3), eqn (2.4), eqn (2.6) and eqn (2.7) can be combined to give:

$$\begin{aligned} &1.803\text{FA} + 2.333\text{GG} \\ &= 0.333\text{P} + 0.333\text{L} + 1.0\text{TCA} + 0.5\text{FA} + 0.833\text{P} + 0.35\text{FA} \\ &\quad +1.25\text{TCA} - 0.833\text{GG} + 1.25\text{FA} + 5.75\text{TCA} \end{aligned} \quad (2.8)$$

This allows TCA $CO_2$ formation to be calculated as:

$$\text{TCA} = 0.396\text{GG} - 0.037\text{FA} - 0.146\text{P} - 0.041\text{L} \quad (2.9)$$

Since total $CO_2$ formation = PPP $CO_2$ + PDH $CO_2$ + TCA cycle $CO_2$ (1.2), PPP $CO_2$ formation = 0.26FA and PDH $CO_2$ = 0.5×(FA + TCA), total $CO_2$ formation can be calculated:

$$CO_2 = 0.70\text{FA} + 0.59\text{GG} - 0.22\text{P} - 0.06\text{L} \quad (2.10)$$

Substituting this into eqn (1.1):

$$\text{Glucose utilization} = 1.70\text{FA} + 1.59\text{GG} + 0.78\text{P} + 0.94\text{L} \quad (2.11)$$

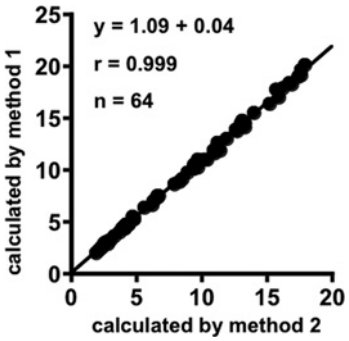


**Figure S6 Comparison of Methods 1 and 2 to calculate rates of glucose utilization by rat adipocytes**

Values for glucose utilization by Method 1 [eqn (1.8)] and by Method 2 [eqn (2.11)] were calculated from values for FA + GG + L + P obtained in experiments with rat adipocytes as described by [4]. Each data point is the mean of four or five independent experiments. For adipocytes from fed rats incubations contained 1 mM glucose with various concentrations of sodium palmitate, adrenaline or dibutyryl cAMP or contained 5 mM glucose and insulin (0.2 $\mu$M) with the same various concentrations of sodium palmitate, adrenaline or dibutyryl cAMP. For adipocytes from 24 h- or 72 h-fasted rats the incubations contained 5 mM glucose and insulin (0.2 $\mu$M) with various concentrations of sodium palmitate.

Eqn (2.11) provides Method 2 for calculation of glucose utilization. Although this method gave an estimate of glucose utilization that was slightly lower than that provided by Method 1 (Figure S6), these two methods were in close qualitative agreement when they were used to assess percentage effects of pamitate and adrenaline on glucose utilization (Figure S7). This provided assurance that the following expressions can reasonably be based on the Method 2 approach.

## TCA cycle $CO_2$ formation

See eqn (2.9).

## Cytosolic NADH balance (F − U)

Substituting eqn (2.9) for the TCA value in eqn (2.5) gives:

$$(\text{F} - \text{U}) = 0.26\text{P} + 0.122\text{FA} - 0.135\text{GG} - 0.02\text{L} \quad (2.12)$$

## Glycolytic and mitochondrial ATP formation

ATP formation by glycolytic substrate level phosphorylation is the quantity in eqn (2.4) minus the value (0.5TCA) for TCA cycle substrate level phosphorylation, i.e.:

$$\begin{aligned} &\text{Glycolytic ATP formation} \\ &= 0.333\text{P} + 0.333\text{L} + 0.5\text{FA} + 0.5\text{TCA} \end{aligned} \quad (2.13)$$

ATP formation by mitochondrial processes is the difference between ATP utilization shown in eqn (2.3) and the glycolytic ATP formation shown in eqn (2.13).

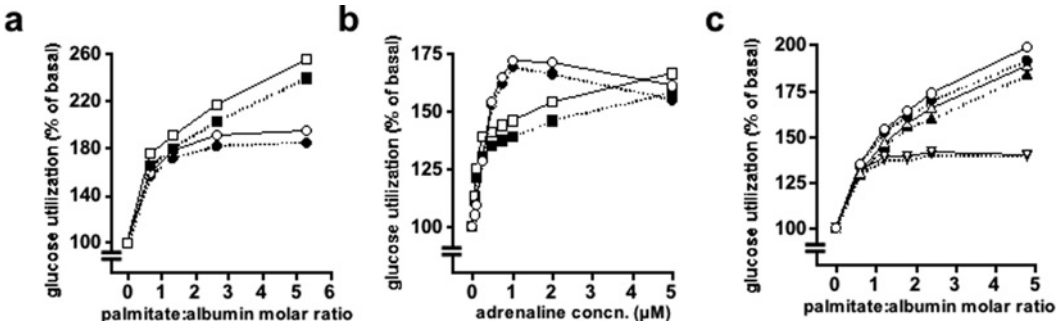


**Figure S7** **Methods 1 and 2 to calculate rates of glucose utilization by rat adipocytes closely agreed when effects of palmitate, adrenaline or fasting were compared on a percentage basis**
The data are from the same experiments as in Figure S6. Open symbols: glucose utilization calculated by Method 1 [eqn (1.8)]. Filled symbols: glucose utilization calculated by Method 2 [eqn (2.11)]. Squares: 1 mM glucose, fed state. Circles: 5 mM glucose + insulin, fed state. Triangles: 5 mM glucose + insulin, 24h-fasted. Inverted triangles: 5 mM glucose + insulin, 72 h-fasted.

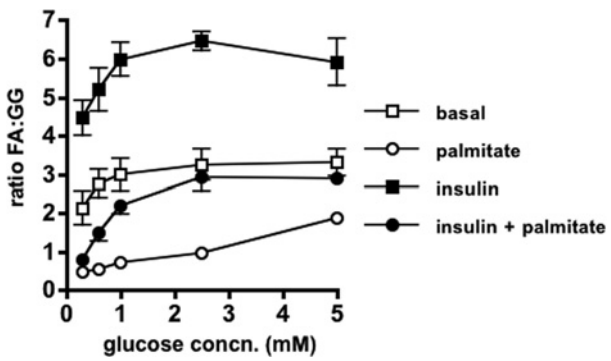


**Figure S8 The effect of palmitate on the FA:GG ratio at different glucose concentrations**
FA and GG values are from the original laboratory data for Figures 1 and 2 of [4]. Adipocytes ($n = 4$) were incubated for 1 h in 1% (w/v) BSA with the indicated concentrations of [U-$^{14}$C]glucose with or without 0.5 mM sodium palmitate or 0.2 $\mu$M insulin.

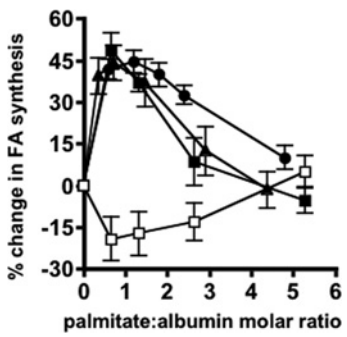


**Figure S9 The effect of palmitate concentration on the conversion of glucose to fatty acids**
The values are derived from the original laboratory data for the following sections of [4] in which adipocytes were incubated for 1 h either with 1 mM glucose (open symbols) or with 5 mM glucose + 0.2 $\mu$M insulin (filled symbols). Squares: from Table 3; 5% BSA; sodium palmitate from 0.5 to 4.0 mM; $n = 5$. Circles: from Figure S3; 2.75% BSA; sodium palmitate from 0.25 to 2.0 mM; $n = 5$. Triangles: from Table 7; 4.5% BSA; sodium palmitate from 0.25 to 3.0 mM; $n = 4$. Palmitate:albumin ratios are shown in order to correct for the different BSA concentrations that were used in these three experiments. In the presence of 5 mM glucose and insulin fatty acid synthesis was significantly increased at all palmitate:albumin ratios below 2.0 ($P$ values ranging from $<0.025$ to $<0.0005$).

## Mitochondrial NADH generation by PDH and the TCA cycle

$$\text{The NADH yield from PDH} = 0.5 \times (\text{FA} + \text{TCA}) \quad (2.14)$$

$$\text{The yield of NADH from the TCA cycle} = 1.5\text{TCA} \quad (2.15)$$

Therefore:

$$\text{total mitochondrial generation of NADH} = 0.5\text{FA} + 2.0\text{TCA} \quad (2.16)$$

### Cytosolic usage of NADH

This is via LDH (lactate dehydrogenase), glycerol 3-phosphate dehydrogenase and via NAD-malate dehydrogenase to provide the contribution by NADP-malate dehydrogenase of NADPH for FA synthesis (see above).

$$\text{Cytosolic NADH usage} = 0.333\text{L} + 0.333\text{GG} + 0.36\text{FA} \quad (2.17)$$

# RESULTS

### Contents of Figures S8 and S9 and Tables S1 and S2

All of the values shown were derived from the original laboratory data of [4]. The contents of these Figures and Tables are

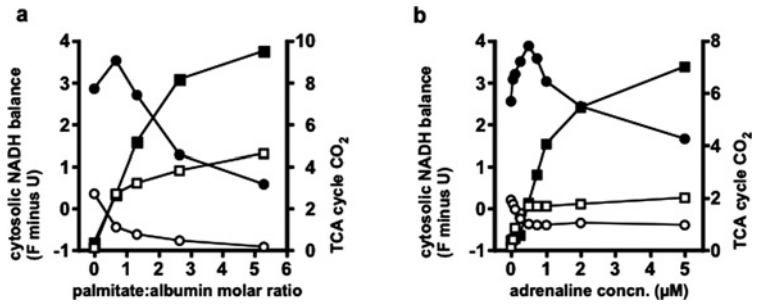


**Figure S10** **The effects of palmitate and adrenaline on rat adipocyte cytosolic NADH balance and TCA cycle $CO_2$ formation**
The values were derived from the original laboratory data of [4] and were calculated using eqn (2.5) and eqn (2.9) for cytosolic NADH balance and TCA cycle activity, respectively. (**a**) Effects of palmitate [0.5–4 mM in the presence of 5% (w/v) BSA]. (**b**) Effects of adrenaline (0.05–5 $\mu$M). Open symbols: 1 mM glucose; filled symbols: 5 mM glucose + insulin (0.2 $\mu$M). Circles: cytosolic NADH balance (F − U) as $\mu$mol of NADH/h/100 $\mu$g cell DNA; squares: TCA cycle $CO_2$ as $\mu$g atoms of C/h/100 $\mu$g cell DNA.

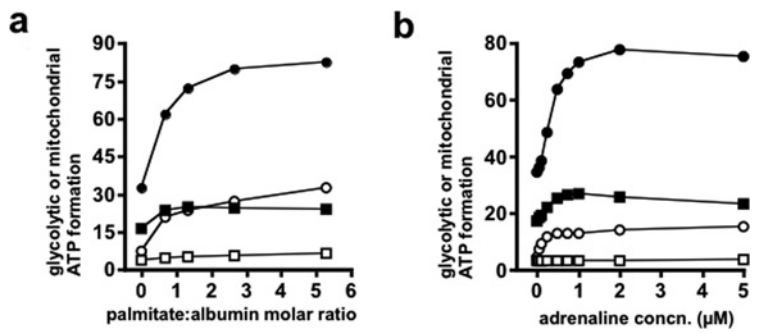


**Figure S11** **The effects of palmitate and adrenaline on rat adipocyte glycolytic and mitochondrial ATP formation**
The values were calculated from the same experimental data as used for Figure S10. Glycolytic ATP formation [eqn (2.13)] was subtracted from total ATP formation [eqn (2.3)] to give mitochondrial ATP formation. (**a**) Effects of palmitate [0.5–4 mM in the presence of 5% (w/v) BSA]. (**b**) Effects of adrenaline [0.05–5 $\mu$M in the presence of 5% (w/v) BSA]. Open symbols: 1 mM glucose; filled symbols: 5 mM glucose + insulin (0.2 $\mu$M). Squares: glycolytic ATP formation; circles: mitochondrial ATP formation. Values are $\mu$mol of ATP/h/100 $\mu$g cell DNA.

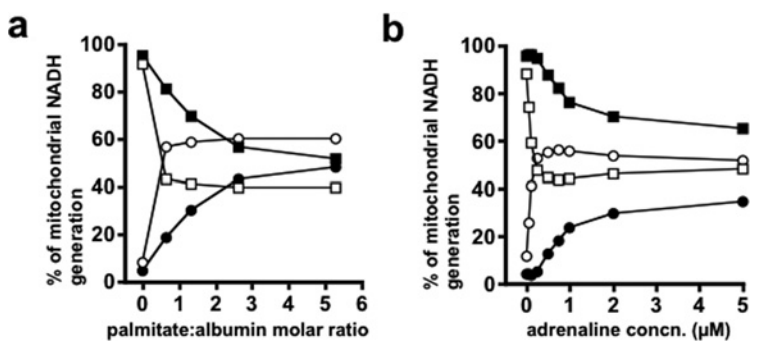


**Figure S12** **The effects of palmitate and adrenaline on the proportions of mitochondrial NADH that are generated by PDH and the TCA cycle in rat adipocytes**
The values were calculated from the same experimental data as used for Figure S10. The NADH yields from PDH [eqn (2.14)] and the TCA cycle [eqn (2.15)] are expressed as percentages of the total mitochondrial generation of NADH [eqn (2.16)]. (**a**) Effects of palmitate [0.5–4 mM in the presence of 5% (w/v) BSA]. (**b**) Effects of adrenaline [0.05–5 $\mu$M in the presence of 5% (w/v) BSA]. Open symbols: 1 mM glucose; filled symbols: 5 mM glucose + insulin (0.2 $\mu$M). Squares: PDH; circles: TCA cycle.

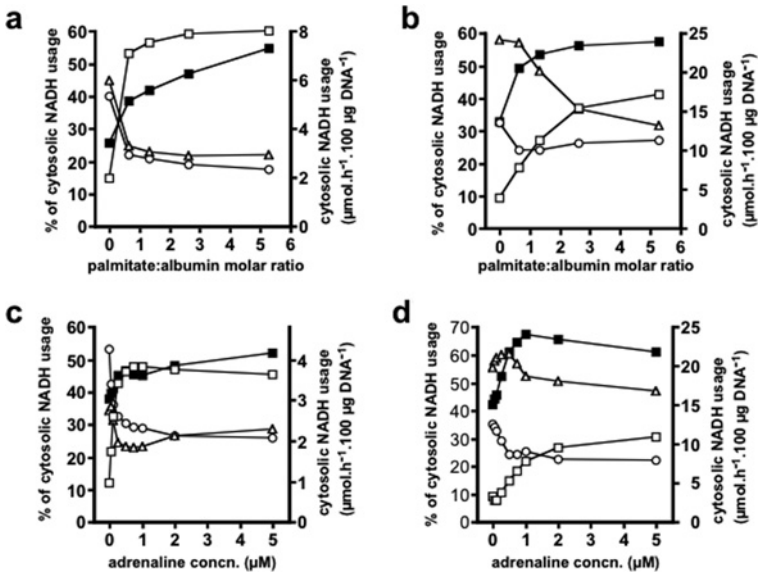


**Figure S13** **The effects of palmitate and adrenaline on the usage of cytosolic NADH for the formation of GG, FA and lactate in rat adipocytes**

The values were calculated from the same experimental data as used for Figure S10. Cytosolic NADH usage was calculated by eqn (2.17) together with the percentages of this due to formation of GG, FA or lactate. Effects of palmitate (0.5–4 mM) or of adrenaline (0.05–5 $\mu$M) both in the presence of 5% (w/v) BSA are shown in (**a**,**b**) and (**c**,**d**) respectively. Filled squares: total cytosolic NADH usage. Open symbols: squares, triangles and circles for the percentage of NADH usage for formation of GG, FA and lactate respectively.

discussed in the main text. Comparison between the present study and the previous study of [4] was judged to be appropriate for the following reasons. Both studies used rats from the same animal colony, used the same procedure for adipocyte isolation and used similar conditions of incubation (bath shaking speed and 1 h of incubation). In the present study addition of 1 mM palmitate (palmitate:albumin ratio = 3.3) increased 5 mM glucose conversion into total lipid (FA + GG) by $124 \pm 21$% ($n = 18$) and by $89 \pm 15$% ($n = 7$) in the absence and presence of insulin respectively, whereas pooled data from three different experiments of [4] showed that addition of palmitate to a palmitate:albumin ratio of 2.4 ($n = 5$), 2.6 ($n = 4$) or 3.3 ($n = 4$) increased 5 mM glucose conversion into total lipid by $198 \pm 24$% ($n = 4$) without insulin and by $92 \pm 7$% ($n = 13$) with insulin. Increases in conversion of 5 mM glucose into total lipid due to addition of insulin were: this study, $164 \pm 18$% ($n = 7$); study of [4], $171 \pm 25$% ($n = 8$). We also compared percentage changes in net glucose utilization, either measured directly in the present study or from [4] as calculated from FA, GG and (lactate + pyruvate) data by Method 1 [eqn (1.8)]. Increases in 5 mM glucose utilization due to addition of palmitate in the presence of insulin were: this study, $60 \pm 8$% ($n = 7$); study of [4], $82 \pm 4$% ($n = 9$). Increases in 5 mM glucose utilization due to the addition of insulin in the absence of palmitate were: this study, $162 \pm 21$% ($n = 7$); study of [4], $117 \pm 21$% ($n = 6$). We also noted that glucose metabolism per incubation flask was similar between the studies under similar experimental conditions.

## Metabolic relationships in adipocyte glucose metabolism based on the Method 2 equations

These are presented in Figures S10–S14. All values are calculated from Tables 3 and 4 of [4] which described experiments where rat adipocytes were incubated for 1 h with 5% (w/v) BSA and additions of [U-$^{14}$C]glucose (either 1 mM alone or 5 mM + insulin), sodium palmitate or adrenaline as indicated in the figure legends. Each data point is the mean of five and four independent experiments with palmitate and adrenaline respectively.

Figure S10 demonstrates the following. (i) Both palmitate and adrenaline substantially increased metabolism of glucose-derived carbon through the TCA cycle. (ii) At 1 mM glucose in the absence of palmitate or adrenaline the cytosolic production of NADH slightly exceeded its utilization giving a small positive (F − U) value. Both palmitate and adrenaline drove (F − U) towards a negative value, i.e. net transfer of reducing equivalents from mitochondria to cytosol would have to occur. (iii) With 5 mM glucose + insulin the (F − U) value was always positive, i.e. net transfer of reducing equivalents from cytosol to mitochondria would occur. Both palmitate and adrenaline caused an increase in the (F − U) value at low concentrations and a decrease at higher concentrations.

Figure S11 demonstrates the following. (i) Both palmitate and adrenaline substantially increased ATP formation; particularly by mitochondrial processes. (ii) Cells incubated with 5 mM glucose and insulin had a greater capacity to provide this extra ATP from glycolysis than did cells incubated with 1 mM glucose.

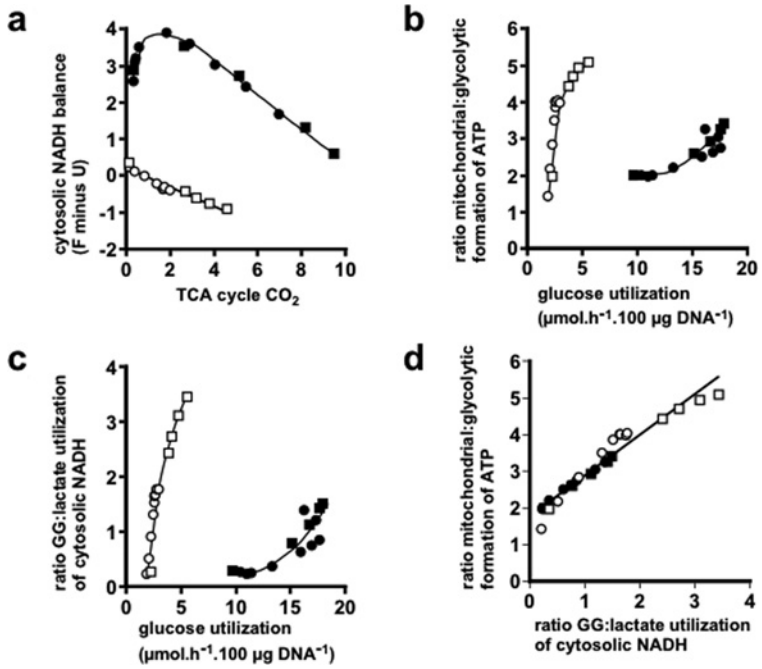


**Figure S14** **Similarities between palmitate and adrenaline in their effects on the metabolism of glucose in rat adipocytes**
Open symbols: 1 mM glucose; filled symbols: 5 mM glucose + insulin (0.2 $\mu$M); squares, palmitate; closed circles, adrenaline. (**a**) Values for TCA cycle $CO_2$ and (F − U) are from Figures S10(a) and S10(b). A linear regression line ($y = -0.25x + 0.21$; $r = -0.962$; $P < 0.001$) is drawn through the 1 mM glucose data points and an arbitrary line is drawn through the 5 mM glucose data points. (**b**) Values for the ratio mitochondrial:glycolytic ATP formation were calculated from the data in Figures S13(a) and S13(b). Glucose utilization was calculated by eqn (2.11) from the FA, GG, L and *P* values in Tables 3 and 4 of [4]. The lines drawn through the data points are arbitrary ones. (**c**) Values for cytosolic NADH usage are from Figures S11(a)–S11(d). Glucose utilization was calculated as in (**b**). The lines drawn through the data points are arbitrary ones. (**d**) The ratios are taken from (**b**) and (**c**). A linear regression line ($y = 1.11x + 1.76$; $r = 0.972$; $P < 0.001$) is drawn through the data points.

Figure S12 shows that both palmitate and adrenaline decreased the generation of NADH by PDH while increasing TCA cycle NADH generation. Steeper concentration dependencies of these effects were seen with 1 mM glucose than with 5 mM glucose + insulin.

The reduction of dihydroxyacetone phosphate to glycerol 3-phosphate that is used in the formation of GG requires cytosolic NADH. Figure S13 shows that the total utilization of cytosolic NADH was increased by both palmitate and adrenaline and that the increased usage of NADH for GG formation was accompanied by decreased usage for lactate formation and, indirectly, for FA synthesis. At 1 mM glucose the concentration dependences of cytosolic NADH usages for lactate and FA formation were very similar in response to palmitate (Figure S13a) and in response to adrenaline (Figure S13c). By contrast, at 5 mM glucose with insulin decreased usage of cytosolic NADH for lactate formation was seen at lower concentrations of palmitate or adrenaline than were needed to cause a decrease in NADH usage for FA synthesis (Figures S13b and S13d). The observation in Figure S10 that palmitate and adrenaline cause the cytosolic NADH balance (F − U) to become negative at 1 mM glucose but not at 5 mM glucose with insulin is relevant in this regard. Presumably at 1 mM glucose cytosolic NADH is in shorter supply and has partially to be provided by transfer of reducing equivalents from mitochondria to the cytosol.

The provision of exogenous palmitate to adipocytes mimics the 'natural' routes of NEFA delivery to the cells; namely from exogenous sources via lipoprotein lipase and from endogenous TAG stores via lipolysis. In the discussion section of the paper, we consider the question of whether endogenous and exogenous NEFA would have identical metabolic effects. In consideration of this question we made the arbitrary plots shown in Figure S14. These allowed data from experiments with palmitate and adrenaline to be normalized to each other in various ways. In all four plots the data for the palmitate and the adrenaline experiments closely superimposed upon each other. In three instances (Figures S14a–S14c) the profiles with 1 mM glucose were very distinct from those with 5 mM glucose + insulin but in Figure S14(d) all of the data appeared to fall on the same curve. We suggest that this normalization approach supports the notion that the effects upon glucose metabolism of exogenous and endogenous NEFA cannot be distinguished from each other. Figure S14(b), besides according with the observation that palmitate and adrenaline increased adipocyte ATP formation/utilization (Figure S11), also

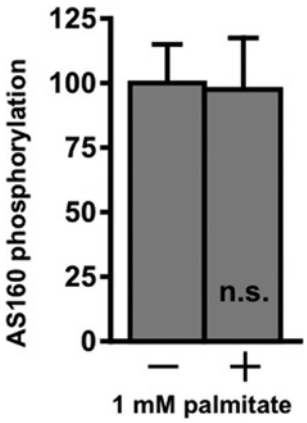


**Figure S15 Palmitate did not alter the phosphorylation of adipocyte AS160**

Proteins in cell lysates used for AMPK activity measurements were separated by SDS/PAGE in 4–20% Bis-Tris gels and then transferred to PVDF membranes. These were probed with phospho-(Ser/Thr) Akt substrate antibody [PAS (periodate–Schiff) antibody] followed by detection by chemiluminescence using horseradish-conjugated secondary antinody. The blots were then stripped at 50°C for 30 min in 62.5 mM Tris/HCl buffer (pH 6.8) containing mercaptoethanol (0.8%, w/v) and SDS (2%, w/v, followed by re-probing with primary antibody against total AS160 protein. Primary and secondary antibodies (from Cell Signaling Technology) were used at a 1:1000 dilution in 20 mM Tris/HCl buffer (pH 7.6) containing 137 mM NaCl, BSA (50 mg/ml) and Tween-20 (0.1%, v/v). Phospho-AS160 measurements were normalized against measurements of total AS160 protein and then expressed as percentages of the values in the absence of palmitate. n.s. indicates $P > 0.05$ for the effect of palmitate ($n = 4$).

particularly accentuated the fact that at 1 mM glucose glycolytic ATP was not able to make much contribution to the extra ATP presumably because of the diversion of much of the increased entry of glucose to GG formation. This supports the supposition in the introduction to the paper that glucose transport at 1 mM glucose availability has greater control strength over glucose metabolism than at 5 mM glucose with insulin also present.

**Table S1 The effects of palmitate at fixed glucose concentrations on the conversion of glucose into FA and GG (glyceride glycerol)**

Values for FA and GG are from the original laboratory data for Table 3 of [4] and are for five independent adipocyte preparations from fed rats. Glucose utilization was calculated by Method 1 [eqn (1.8)]. Adipocytes were incubated for 1 h with 5% BSA and the indicated concentrations of sodium palmitate. *Conversion rates and differences due to palmitate are expressed as $\mu$g atoms of glucose carbon $\cdot h^{-1} \cdot 100$ $\mu$g of cell $DNA^{-1}$.

(**a**) 1 mM [U-$^{14}$C]glucose without insulin

| | **Glucose conversion into** | | | | | |
|---|---|---|---|---|---|---|
| | **FA** | | | **GG** | | |
| **Palmitate concentration (mM)** | **Conversion rate* and percentage of glucose utilization** | **Difference*** | **% Change** | **Conversion rate* and percentage of glucose utilization** | **Difference*** | **% Change** |
| 0 | 4.28 ± 1.40, 26.5 ± 3.6% | 0 | 0 | 1.54 ± 0.26, 10.5 ± 1.0% | 0 | 0 |
| 0.5 | 3.51 ± 1.46, 11.6 ± 2.8% | − 0.77 ± 0.12, $P < 0.005$ | − 18 | 8.20 ± 1.24, 32.0 ± 2.2% | 6.66 ± 1.00, $P < 0.005$ | 432 |
| 1.0 | 3.54 ± 1.53, 10.5 ± 2.8% | − 0.74 ± 0.18, $P < 0.025$ | − 17 | 9.46 ± 1.44, 33.1 ± 1.8% | 7.92 ± 1.21, $P < 0.005$ | 514 |
| 2.0 | 3.74 ± 1.55, 10.0 ± 2.5% | − 0.54 ± 0.17, $P < 0.05$ | − 13 | 11.1 ± 1.50, 34.6 ± 2.0% | 9.57 ± 1.29, $P < 0.005$ | 621 |
| 4.0 | 4.48 ± 1.86, 10.2 ± 2.4% | 0.20 ± 0.48, N.S. | 5 | 13.3 ± 1.90, 35.2 ± 2.1% | 11.7 ± 1.70, $P < 0.005$ | 762 |

(**b**) 5 mM [U-$^{14}$C]glucose with insulin (0.2 $\mu$M)

| | **Glucose conversion into** | | | | | |
|---|---|---|---|---|---|---|
| | **FA** | | | **GG** | | |
| **Palmitate concentration (mM)** | **Conversion rate* and percentage of glucose utilization** | **Difference*** | **% Change** | **Conversion rate* and percentage of glucose utilization** | **Difference*** | **% Change** |
| 0 | 22.0 ± 2.2, 35.4 ± 1.5% | 0 | 0 | 3.82 ± 0.58, 6.1 ± 0.7% | 0 | 0 |
| 0.5 | 32.6 ± 3.5, 32.9 ± 1.2% | 10.6 ± 1.8, $P < 0.005$ | 48 | 11.5 ± 1.0, 11.8 ± 1.2% | 7.7 ± 0.7, $P < 0.005$ | 202 |
| 1.0 | 30.2 ± 3.3, 27.2 ± 1.1% | 8.2 ± 1.3, $P < 0.005$ | 37 | 18.2 ± 1.8, 16.7 ± 1.6% | 14.4 ± 1.5, $P < 0.005$ | 377 |
| 2.0 | 24.0 ± 3.4, 20.0 ± 1.3% | 2.0 ± 1.1, N.S. | 9 | 26.1 ± 2.6, 22.4 ± 2.5% | 22.3 ± 2.2, $P < 0.005$ | 584 |
| 4.0 | 21.0 ± 2.6, 17.1 ± 0.8% | − 1.0 ± 1.3, N.S. | − 5 | 29.5 ± 3.0, 24.5 ± 2.2% | 25.7 ± 2.5, $P < 0.005$ | 673 |

**Table S2 Effects of a fixed concentration of palmitate on glucose conversion into FA and GG at different glucose concentrations**

Values for FA and GG are from the original laboratory data for Figures 1 and 2 of [4] and are for four independent adipocyte preparations from fed rats. Adipocytes were incubated for 1 h with 1.0% BSA and the indicated concentrations of [U-$^{14}$C]glucose with or without 0.5 mM sodium palmitate. *Conversion rates and differences due to palmitate are expressed as $\mu$g atoms of glucose carbon $\cdot h^{-1} \cdot 100$ $\mu$g of cell DNA$^{-1}$.

(**a**) Without insulin

| | Glucose conversion into | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FA | | | | GG | | | |
| | Conversion rate* | | | | Conversion rate* | | | |
| Glucose concentration (mM) | 'Basal' | + Palmitate | Difference* | % Change | 'Basal' | + Palmitate | Difference* | % Change |
| 0.3 | 1.07 ± 0.13 | 0.85 ± 0.08 | − 0.22 ± 0.05, $P < 0.025$ | − 21 | 0.55 ± 0.08 | 1.82 ± 0.15 | 1.27 ± 0.11, $P < 0.005$ | 230 |
| 0.6 | 2.27 ± 0.23 | 1.48 ± 0.37 | − 0.79 ± 0.22, $P < 0.025$ | − 35 | 0.86 ± 0.09 | 3.18 ± 0.24 | 2.32 ± 0.16, $P < 0.0005$ | 270 |
| 1.0 | 3.23 ± 0.39 | 3.03 ± 0.31 | − 0.20 ± 0.09, N.S. | − 6 | 1.11 ± 0.09 | 4.25 ± 0.32 | 3.14 ± 0.27, $P < 0.005$ | 283 |
| 2.5 | 4.73 ± 0.49 | 5.71 ± 0.40 | 0.98 ± 0.11, $P < 0.005$ | 21 | 1.47 ± 0.08 | 5.94 ± 0.37 | 4.47 ± 0.29, $P < 0.0005$ | 304 |
| 5.0 | 6.21 ± 0.53 | 15.3 ± 0.7 | 9.10 ± 0.79, $P < 0.005$ | 146 | 1.91 ± 0.12 | 8.49 ± 0.92 | 6.58 ± 0.79, $P < 0.005$ | 345 |

(**b**) With insulin (0.2 $\mu$M)

| | Glucose conversion into | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | FA | | | | GG | | | |
| | Conversion rate* | | | | Conversion rate* | | | |
| Glucose concentration (mM) | 'Basal' | + Palmitate | Difference* | % Change | 'Basal' | + Palmitate | Difference* | % Change |
| 0.3 | 5.21 ± 0.19 | 3.26 ± 0.37 | − 1.95 ± 0.17, $P < 0.005$ | − 37 | 1.20 ± 0.08 | 4.24 ± 0.19 | 3.04 ± 0.11, $P < 0.0005$ | 253 |
| 0.6 | 10.7 ± 0.3 | 9.22 ± 0.70 | − 1.48 ± 0.37, $P < 0.025$ | − 14 | 2.11 ± 0.17 | 6.47 ± 0.62 | 4.36 ± 0.46, $P < 0.005$ | 207 |
| 1.0 | 15.7 ± 0.7 | 17.4 ± 0.8 | 1.7 ± 0.8, N.S. | 11 | 2.66 ± 0.22 | 8.12 ± 0.44 | 5.46 ± 0.27, $P < 0.0005$ | 205 |
| 2.5 | 21.1 ± 0.7 | 25.1 ± 1.6 | 4.0 ± 1.3, $P < 0.025$ | 19 | 3.29 ± 0.20 | 9.50 ± 0.48 | 6.21 ± 0.47, $P < 0.0005$ | 189 |
| 5.0 | 20.9 ± 1.2 | 29.6 ± 0.7 | 8.7 ± 0.6, $P < 0.0005$ | 42 | 3.65 ± 0.41 | 10.3 ± 0.4 | 6.67 ± 0.24, $P < 0.0005$ | 183 |

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